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Phase III randomised trial

Necrosis predicts benefit from hypoxia-modifying therapy in patients with high risk bladder cancer enrolled in a phase III randomised trial

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ABSTRACT

Background and purpose: Addition of carbogen and nicotinamide (hypoxia-modifying agents) to radiotherapy improves the survival of patients with high risk bladder cancer. The study investigated whether histopathological tumour features and putative hypoxia markers predicted benefit from hypoxia modification.

Materials and methods: Samples were available from 231 patients with high grade and invasive bladder carcinoma from the BCON phase III trial of radiotherapy (RT) alone or with carbogen and nicotinamide (RT + CON). Histopathological tumour features examined were: necrosis, growth pattern, growing margin, and tumour/stroma ratio. Hypoxia markers carbonic anhydrase-IX and glucose transporter-1 were examined using tissue microarrays.

Results: Necrosis was the only independent prognostic indicator ($P = 0.04$). Necrosis also predicted benefit from hypoxia modification. Five-year overall survival was 48% (RT) versus 39% (RT + CON) ($P = 0.32$) in patients without necrosis and 34% (RT) versus 56% (RT + CON) ($P = 0.004$) in patients with necrosis. There was a significant treatment by necrosis strata interaction ($P = 0.001$ adjusted). Necrosis was an independent predictor of benefit from RT + CON versus RT (hazard ratio [HR]: 0.43, 95% CI 0.25–0.73, $P = 0.002$). This trend was not observed when there was no necrosis (HR: 1.64, 95% CI 0.95–2.85, $P = 0.08$).

Conclusions: Necrosis predicts benefit from hypoxia modification in patients with high risk bladder cancer and should be used to select patients; it is simple to identify and easy to incorporate into routine histopathological examination.

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Muscle invasive bladder cancer has five-year survival rates of 50–60%. Treatment is with radical cystectomy or bladder-sparing protocols involving radiotherapy. Both show similar survival rates [1,2] with the latter preserving a functional bladder in three quarters of patients [3]. The BCON (bladder carbogen and nicotinamide) phase III clinical trial showed the addition of carbogen and nicotinamide (CON) to radiotherapy (RT) improved overall survival (OS) [4]. Not all patients benefit from the additional hypoxia-modifying therapy and there is a need for biomarkers to improve the individualisation of bladder cancer treatment.

There is evidence in head and neck cancer that hypoxic tumours benefit most from hypoxia-modifying therapy [5–10]. Similar evidence is not available for bladder cancers but, like other solid tumours, they contain hypoxic areas and the expression of high levels of hypoxia-inducible markers is associated with a poor prognosis [11–13].

There is no means to routinely measure tumour hypoxia in the clinic. Once considered the gold standard, the Eppendorf pO_2 histograph is no longer available, endogenous protein markers suffer poor specificity [14] and large intra-tumour variation [15], and exogenous nitroimidazole markers require intravenous/oral administration and the need for prospective assessment has limited the number of studies carried out [16]. As a simple histopathology approach would be an advantage, we hypothesised that tumour features such as necrosis, growth pattern, appearance of

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growing margin, or tumour/stroma ratio (TSR) might reflect tumour hypoxia as well as putative hypoxia markers carbonic anhydrase-IX (CA-IX) and glucose transporter-1 (Glut-1).

Tumour necrosis is believed to represent the endpoint of severe, chronic hypoxia distal to functional blood vessels. In bladder cancer, necrosis has been associated with the expression of hypoxia markers hypoxia inducible factor-1 and CA-IX and a poor outcome following primary cystectomy [13]. Tumour growth pattern [17] and appearance of growing margin [18] are also associated with a poor prognosis in urothelial disease. Solid growing tumours may be more hypoxic; they comprise large tumour islands, which may have less well organised vasculature than papillary tumours. An infiltrative tumour margin may indicate the presence of hypoxia, which can drive invasion and metastasis [19]. TSR may also be indicative of tumour hypoxia. Stromal expression of the hypoxia marker, monocarboxylate transporter 4 predicts poor outcome in breast cancer [20] and high levels of tumour stroma (>50%) have been linked to a worse prognosis in several cancer types [21–24]. Expression of CA-IX and Glut-1 was associated with a poor survival in bladder cancer [11], and so may also predict treatment benefit. Therefore, a retrospective study was performed to investigate the ability of the various histopathological features and putative hypoxia markers to predict benefit from hypoxia modification using samples from patients enrolled in the BCON trial. REMARK

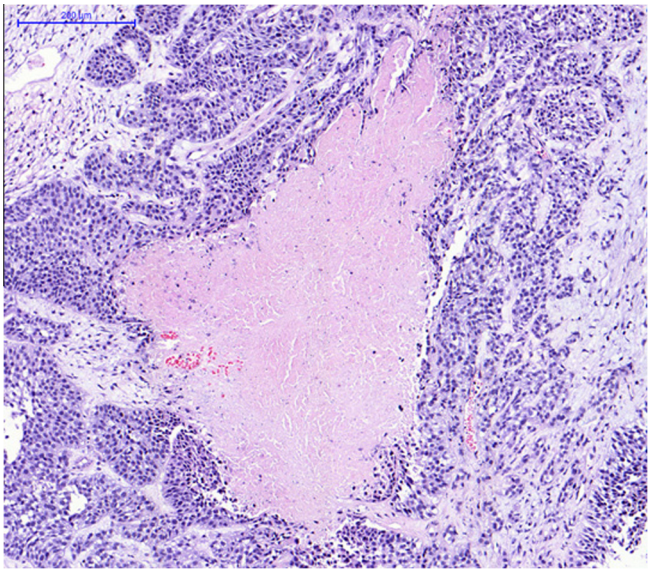

Fig. 2. H&E-stained bladder cancer section with necrosis. Necrosis is identified by the presence of cell ghosts and is eosinophilic and granular. Original magnification 400×.

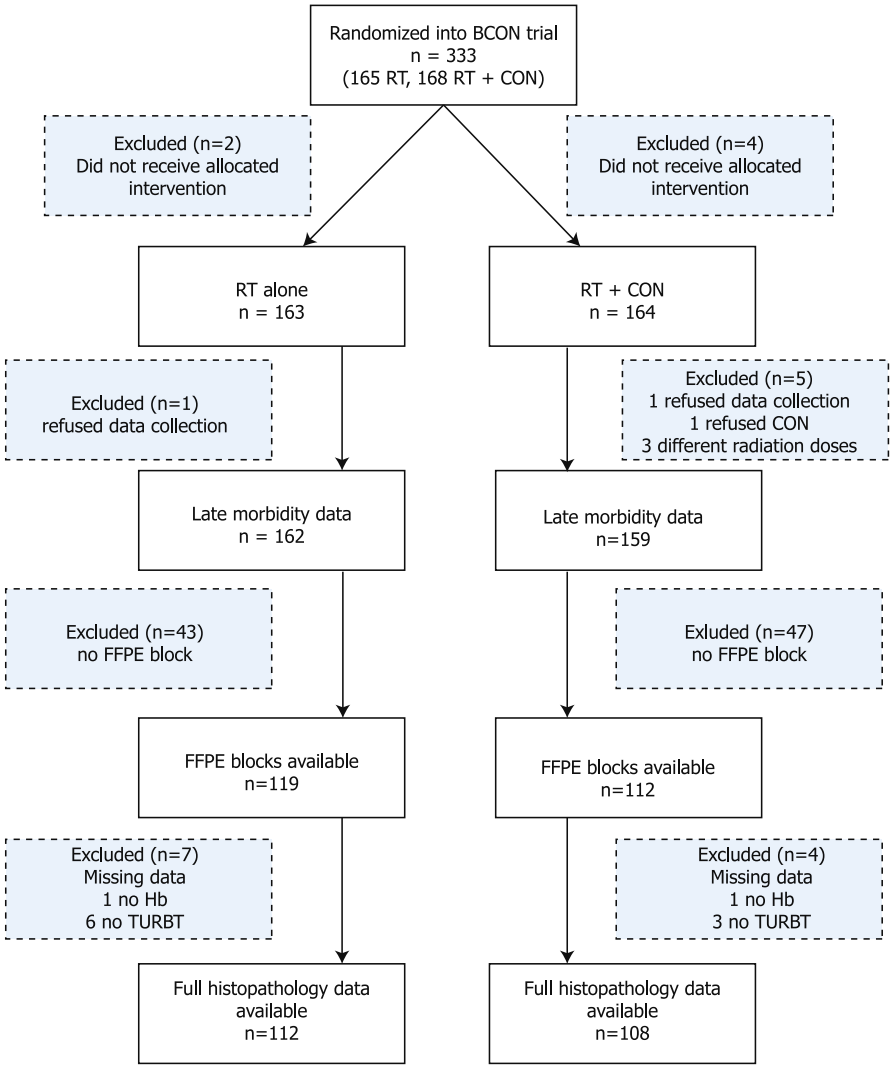

Fig. 1. CONSORT diagram.

Table 1
Clinicopathologic patient details grouped by random assignment.

Variable	RT *N* = 119	RT + CON *N* = 112	*P*
Gender			0.55
Male	93 (78%)	92 (82%)	
Female	26 (22%)	20 (18%)	
Age (years)	75 (51–90)	75 (51–89)	0.54
Tumour stage			0.49
T1	12 (10%)	10 (9%)	
T2	75 (63%)	80 (71%)	
T3	27 (23%)	17 (15%)	
T4a	5 (4%)	5 (4%)	
TURBT			0.89
Complete	47 (39%)	44 (39%)	
Partial	34 (29%)	36 (32%)	
Biopsy	32 (27%)	29 (26%)	
No data	6 (5%)	3 (3%)	
Radiotherapy			0.58
32 fractions	80 (67%)	81 (72%)	
20 fractions	38 (32%)	28 (25%)	
Other	1 (1%)	3 (3%)	
Necrosis			0.83
Present	61 (51%)	60 (54%)	
Absent	58 (49%)	52 (46%)	
Growth pattern			0.24
Papillary	19 (16%)	26 (23%)	
Solid	50 (42%)	49 (44%)	
Both	50 (42%)	37 (33%)	
Growing margin			0.33
Broad	5 (4%)	2 (2%)	
Infiltrative	114 (96%)	109 (97%)	
Both	0	1 (1%)	
TSR			0.33
High $\geqslant$50%	108 (91%)	101 (90%)	
Low <50%	9 (7%)	11 (10%)	
Mixed	2 (1%)	0 (0%)	
Concurrent pTis			0.006
Present	40 (34%)	19 (17%)	
Absent	79 (66%)	93 (83%)	
Hb (g/dL)	13.6 (9.3–16.9)	14.1 (9.5–17.2)	0.29
No data	1 (1%)	1 (1%)	
CA-IX score	4.8 (0–208.4)	4.5 (0–148.9)	0.81
No data	23 (19%)	19 (17%)	
Glut-1 score	110.0 (0–300)	90.9 (0–290.0)	0.61
No data	24 (20%)	22 (20%)	

Data are represented as *n* (%) or median (range). *Abbreviations:* RT, radiotherapy; CON, carbogen and nicotinamide; TURBT, transurethral resection of the bladder tumour; CIS, carcinoma *in situ*; Hb, haemoglobin.

guidelines for reporting prognostic tumour marker studies were followed throughout [25].

Materials and methods

Patients and tissue samples

A retrospective cohort study was carried out in 231 patients with histologically proven high grade urothelial (transitional cell) carcinoma of the bladder, stage T1, T2, T3 or T4a (metastases free). Patients participated in the prospective BCON phase III trial and were randomised between November 2000 and April 2006. Samples from 11 UK hospitals were obtained for 251 of the 333 patients enrolled in BCON. Samples from 20 patients were excluded as they were: low grade [1], squamous cell [2], reclassified as T4b [2], or contained insufficient tumour material [15]. Fig. 1 is the CONSORT diagram. The study was approved by the local research ethics committee (LREC 09/H1013/24) and informed consent for sample collection and analysis before randomisation was mandatory.

Two RT schedules were permitted: 55 Gy in 20 fractions in four weeks and 64 Gy in 32 fractions in 6½ weeks. Treatments were given daily, five times per week and all fields were treated in each session. Carbogen (2% CO_2 and 98% O_2) was administered 5 minutes before and during RT. An oral dose of 40 or 60 mg kg^{-1} nicotinamide (Larkhall Laboratories, Charlbury, UK) was given 1½ to 2 h before each fraction.

Tissue samples were obtained by pre-treatment transurethral resection of the bladder tumour (TURBT; biopsy, partial or complete). Tumour debulking was performed using a diathermy loop, which produced strips of tissue approximately 6 mm in width and of variable length. One block per cm tumour diameter was formalin-fixed and paraffin-embedded (FFPE). Up to eight blocks per patient were examined to assess intra-tumour heterogeneity.

For independent validation of the prognostic ability of necrosis, raw data were obtained from a previously published study [13]. Briefly, samples were obtained from 90 patients who underwent radical cystectomy for invasive and high grade bladder cancer.

Histopathology

One 4 μm haematoxylin and eosin (H&E)-stained section from each FFPE block was analysed. Staging was clinical and pathological (TNM AJCC/UICC classifications). Grading was according to UK Royal College Pathologists (RCP) guidelines (WHO 1973). Tissue hypoxia-related coagulative tumour necrosis is characterised by increased tissue eosinophilia, nuclear breakdown and ultimate loss of tissue architecture (Fig. 2). Following REMARK guidelines, cut-off values were selected from the literature prior to scoring. Necrosis was scored as absent versus present (any amount) as used in routine histopathology reporting for other cancers. Growth pattern was recorded as solid (flat, broad base), papillary (polypoid), or both; growing margin as broad, infiltrative (narrow cords or single cells at invasive front), or both. For TSR, patients with <50% were classified as TSR low and patients with $\geqslant$50% as TSR high. This cut-off was determined to have maximum discriminative power in colon [21], oesophageal [22], and breast [23,24] cancer. As it is a recognised prognostic factor [26], presence of flat carcinoma *in situ* (CIS or pTis) was recorded at the same time. Two consultant histopathologists assessed the slides (HD & SA) as in routine pathology reporting with no double scoring. In case of tumour heterogeneity, those areas with the worst histopathological feature were deemed decisive.

Immunohistochemistry

For tissue microarrays, tumour areas were demarcated by a histopathologist and 1 mm diameter cores (up to 3 per tumour region from 2 regions) were taken. Where possible, cores were also taken from regions of normal urothelium. Up to 120 cores were placed in a single FFPE block in a standardised pattern (MTA-1, Beecher Instruments, Silver Spring, MD). Immunohistochemistry was carried out for CA-IX and Glut-1 as described previously [11]. In a subset of cases, immunohistochemistry for Ki-67 was also performed [11].

Immunohistochemical analysis

CA-IX and Glut-1 were assessed using an H-score – the product of intensity (0–3) and estimated percentage labelling of viable tumour cells in cores (100× magnification), giving a range of 0-300. Ki-67 was scored on a field-by-field basis (400× magnification) as a percentage of viable tumour cells labelled. Two observers (AE & JJI) scored cores independently and a mean score was taken. Independent scores correlated well (Spearman ρ: CA-IX = 0.90, Glut-1 = 0.90, Ki-67 = 0.85). Discordant cases were re-scored by a

Table 2
Hazard ratios for all cause mortality in patients treated with RT alone. All covariates included in the model are also tabulated.

Variable	N_{UV}	HR_{UV}	95% CI	*P*	N_{MV}	HR_{MV}	95% CI	*P*
Gender	119			0.22	87			0.25
Male (reference)	93				70			
Female	26	1.41	0.82–2.42		17	1.57	0.73–3.37	
Age (years)*	119	1.05	1.02–1.08	0.003	87	1.05	1.00–1.09	0.04
Stage	119			0.12	87			0.10
T1 (reference)	12				8			
T2	75	3.09	0.96–9.94		58	6.49	0.82–51.1	
T3	27	2.31	0.66–8.10		18	3.96	0.43–36.5	
T4a	5	3.89	0.78–19.34		3	6.75	0.51–88.7	
TURBT	113			0.90	87			0.59
Complete (reference)	47				33			
Partial	34	0.90	0.49–1.63		29	0.80	0.39–1.65	
Biopsy	32	1.04	0.58–1.87		25	0.66	0.30–1.46	
Necrosis	119			0.01	87			0.04
Absent (reference)	58				38			
Present	61	1.82	1.12–2.96		49	2.11	1.04–4.28	
Growth pattern	119			0.44	87			0.47
Papillary (reference)	19				4			
Solid	50	1.13	0.58–2.20		42	1.94	0.40–9.37	
Both	50	0.80	0.40–1.59		41	1.38	0.27–7.02	
pTis	119			0.03	87			0.02
Absent (reference)	79				56			
Present	40	1.71	1.05–2.77		31	2.18	1.17–4.06	
Hb (g/dL)*	118	0.86	0.73–1.01	0.07	87	0.91	0.73–1.13	0.38
CA-IX score	96			0.10	87			0.78
0 (reference)	31				29			
>0	65	1.62	0.90–2.92		58	1.12	0.52–2.41	
Glut-1 score	95			0.65	87			0.66
<100 (reference)	44				42			
⩾100	51	1.13	0.66–1.92		45	1.15	0.61–2.18	

Abbreviations: HR, hazard ratio; UV, univariate; MV, multivariate; TURBT, transurethral resection of the bladder tumour; pTis, carcinoma *in situ*; Hb, haemoglobin.
* Variables marked with a * were entered as continuous variables and hence the HR is for a unit increase in the variable.

consultant histopathologist (HD) and their score used. Scorers were blinded to clinical outcome data. Tumour cores were not scored if there was <10% viable tumour. The mean (range) number of cores scored were 4.3 (1–6), 4.3 (1–6) and 2.1 (1–3) for CA-IX, Glut-1 and Ki67, respectively. In normal urothelium, median scores (CA-IX and Glut-1) were 0 and median% Ki-67 was 0. An a priori cut-off point of 0 was selected to dichotomise patients as previously described for CA-IX [11,13] and Glut-1 [11]. However, for Glut-1, a cut-off of 100 was then selected retrospectively as it was close to the median value and because only 9 cases (4.9%) were negative.

Statistical analyses

Statistical analyses were performed using S Plus (Insightful Corporation, Seattle, WA). Five-year OS was taken as time from randomisation to death from any cause; patients still alive were censored at the time last seen or at five years whichever was earlier. Local relapse free survival was taken as time to tumour recurrence in bladder (muscle invasive lesions only), locoregional failure or death from any cause. Patients alive and free of local disease were censored at their last follow-up. Patients with persistent muscle invasive or with no cystoscopy following treatment had their time set to zero. Fifty-four patients had a follow up beyond five years, of which 43 were censored. Follow up beyond five years was not routinely reported at all hospital sites. Survival estimates were obtained using the Kaplan–Meier method and differences compared using the Mantel-Cox log-rank test. Hazard ratios (HR) and 95% CI were obtained using Cox's proportional hazard model. Heterogeneity in the treatment effect according to necrosis was addressed within a stratified Cox regression model using appropriate stratum specific-treatment variables. This analysis was performed first just with necrosis, CA-IX or Glut-1 and treatment information and second adjusted for prognostic features. The chi-square test was used to compare proportions across the levels of categorical factors and Yates' correction was used for 2×2 tables; the Mann-Whitney *U* test was used to compare median values for continuous variables between two groups. Spearman correlation coefficients were used to assess statistical associations. All *P*-values were two-sided and significance was set to $P \leqslant 0.05$.

Results

In the subset of 231 BCON patients available for study, 119 received RT alone and 112 RT + CON. Most patients (229; 99%) received ⩾90% of the prescribed RT (i.e. 20 or 32 fractions). In the experimental arm, 100 patients (89%) received ⩾90% of the stipulated carbogen doses and 81 (72%) received ⩾90% of the nicotinamide at the dose of 60 mg/kg. The 231 patients had a median age at randomisation of 75 (range 51–90) years. 185 (80%) patients were male and 46 (20%) female. Stage was T1, T2, T3, T4a in 22 (10%), 155 (67%), 44 (19%) and 10 (4%) patients, respectively. Table 1 lists clinicopathologic details by randomisation arm. Supplementary Table S1 shows the clinicopathologic details for the necrosis cohort were very similar to those for patients enrolled in the main trial.

Tumour necrosis was evident in 121 (52%) patients. Supplementary Table S2 shows the distribution of patients in the necrotic and non-necrotic sub-groups by randomisation arm. As most tumours had an infiltrative growing margin and high TSR (Table 1), these

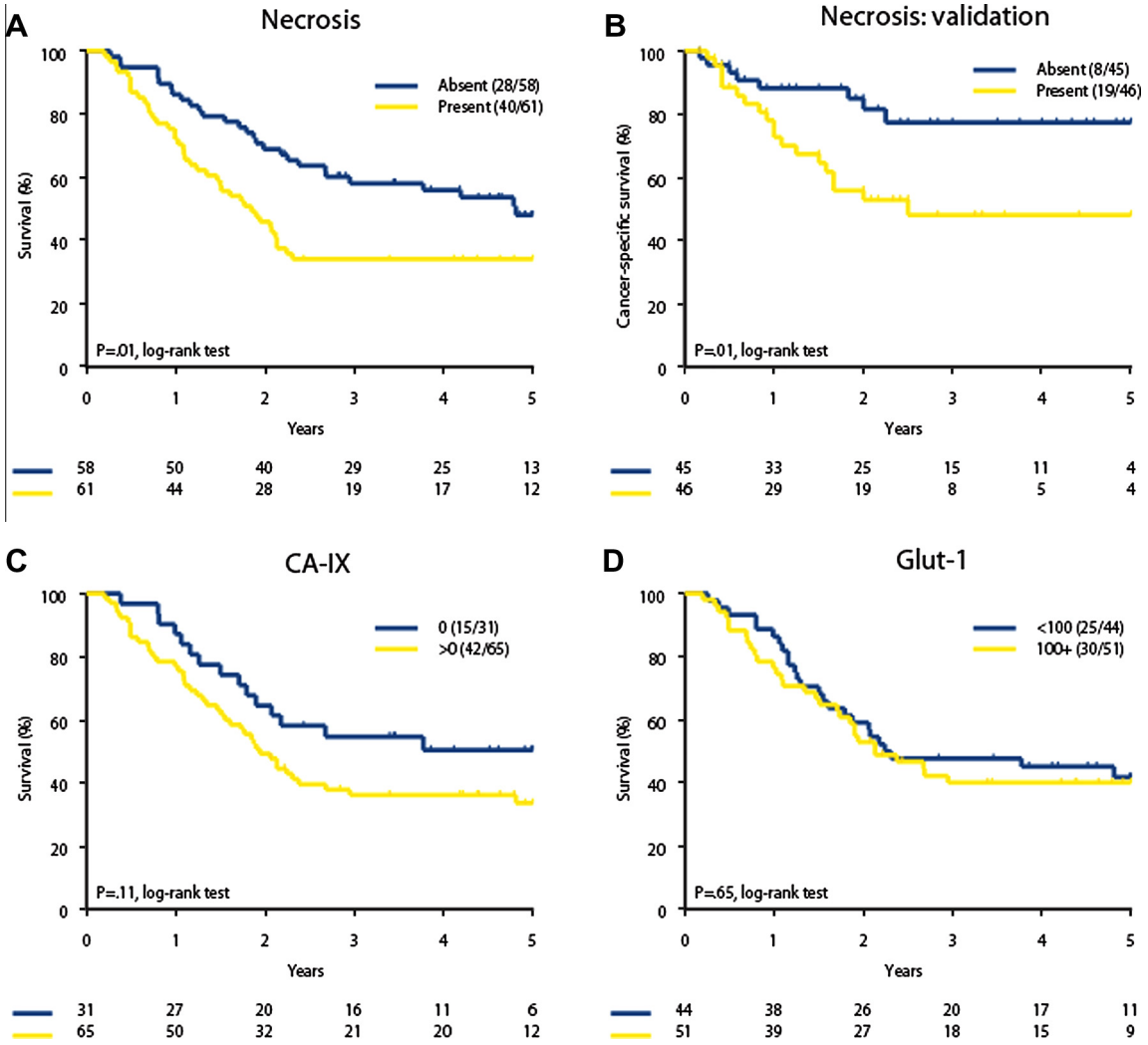

Fig. 3. Kaplan–Meier plots for: (A, C, D) overall survival of BCON patients who received radiotherapy as primary treatment and (B) cancer-specific survival of patients who underwent radical cystectomy as primary treatment [13]. Patients are stratified by the absence or presence of necrosis (A, B), CA-IX score (C) or Glut-1 score (D). Log rank *P* values and number of patients at risk in each yearly interval are also shown.

features were not investigated further. Median CA-IX and Glut-1 scores were 4.7 (range 0–208.4; 189 cases) and 103.9 (range 0–300; 185 cases) respectively. Median proliferative fraction as defined by percentage Ki-67 immunolabelling was 10.4% (range 0–72.8%; 92 cases). There were 63 (33.3%), 9 (4.9%) and 1 (1.1%) tumour(s) that were negative for CA-IX, Glut-1 and Ki-67, respectively.

Analyses were carried out for OS and local relapse free survival but as the results were very similar, the findings for OS only are presented. Prognosis was investigated in 119 patients receiving RT alone as this was defined as standard bladder-sparing treatment. Table 2 summarises the results of univariate and multivariate analyses. Increasing age ($P = 0.003$), presence of necrosis ($P = 0.01$), and concurrent pTis ($P = 0.03$) were the only statistically significant adverse prognostic factors upon univariate analysis. Age ($P = 0.04$), necrosis ($P = 0.04$), and concurrent pTis ($P = 0.02$) retained significance upon multivariate analysis. Fig. 3A shows that necrosis is a significant prognostic factor in high grade and invasive bladder cancer treated with RT alone (log rank $P = 0.01$). Five-year OS was 48% in the absence of necrosis and 34% in the presence of necrosis. Supplementary Fig. S1 shows the result for local relapse free survival (log rank $P = 0.016$). In the same cohort of patients neither CA-IX (Fig. 3C) nor Glut-1 (Fig. 3D) had prognostic significance. Given the strong prognostic significance of necrosis, an independent cohort was obtained. The cohort comprised a published series of bladder cancer patients undergoing radical cystectomy, which showed that necrosis was prevalent in high grade and invasive disease (46/91, 51%). The patient and tumour characteristics for the cohort have already been published [13]. Tumour necrosis scored on whole sections as absent, comedo (<5 mm), or gross (>5 mm) was prognostic (log rank $P < 0.0001$) [13]. The raw data were obtained and necrosis reanalysed as absent versus present as used in our study and in standard histopathology reporting. Fig. 3B shows that necrosis was prognostic for an adverse outcome in the independent cohort. Cancer-specific survival was 82% in the absence and 59% in the presence of necrosis (log rank $P = 0.01$).

As necrosis was the only histopathological feature with prognostic significance, it was the only one studied further for its ability to predict benefit from hypoxia modification. Of the 231 cases, 124 patients died within five years (54%). Median follow-up of the 107 survivors was 5.0 (range 1.7–5.0) years. Five-year OS was 41% for RT alone versus 48% for RT + CON (log rank $P = 0.14$). In 110 patients with no evident necrosis the five-year OS was 48% for RT alone versus 39% for RT + CON (log rank $P = 0.32$) (Fig. 4A). In 121 patients with tumour necrosis the five-year OS was 34% for RT alone versus 56% for RT + CON (log rank $P = 0.004$) (Fig. 4B).

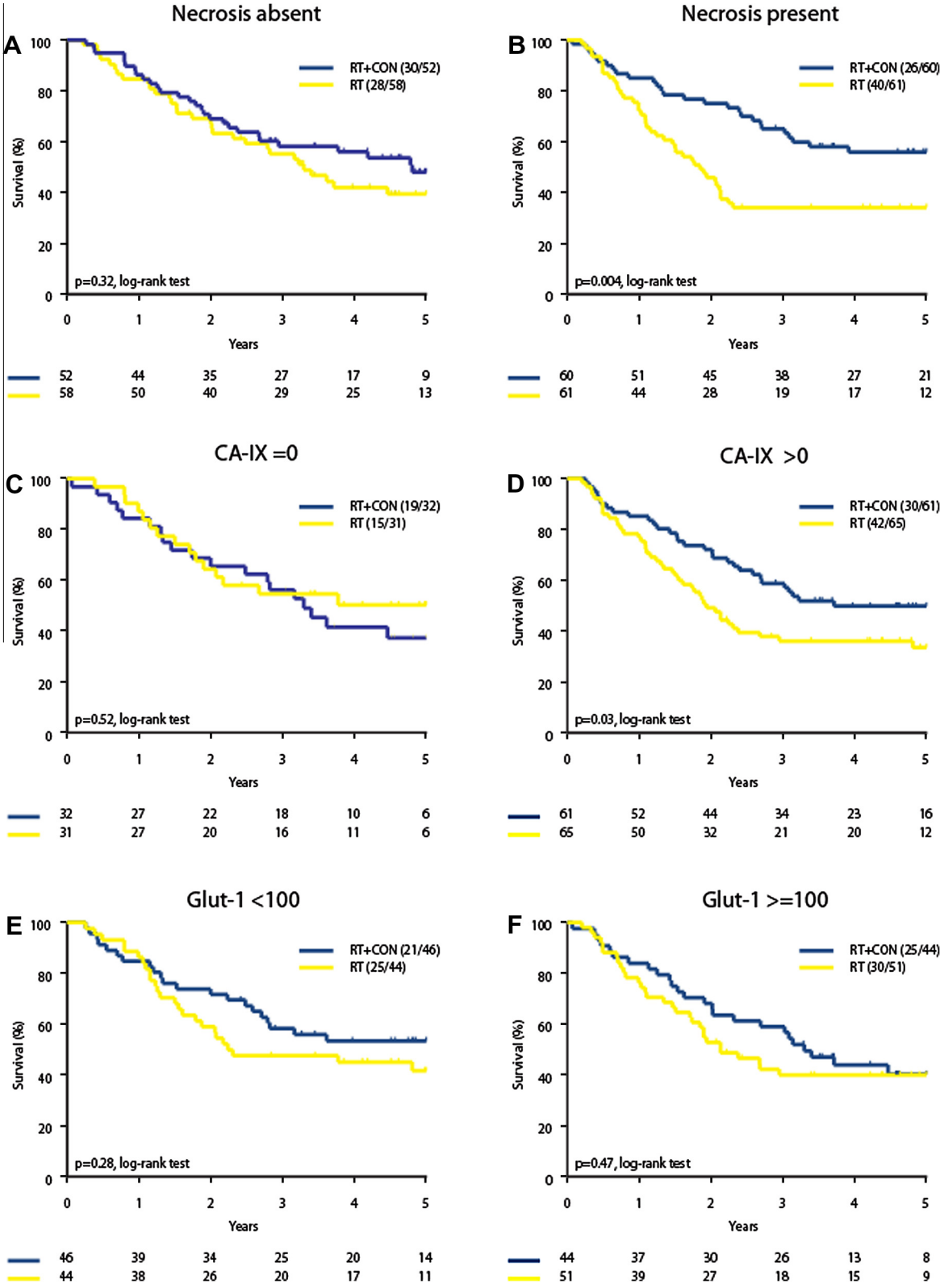

Fig. 4. Kaplan–Meier plots for: overall survival after radiotherapy (RT) alone or with carbogen and nicotinamide (RT + CON) and stratified according to the (A) absence or (B) presence of necrosis, (C) CA-IX score = 0 or (D) >0, or (E) Glut-1 score <100 or (F) ⩾100. Log rank *P* values and number of patients at risk in each yearly interval are also shown.

Supplementary Fig. S1 shows very similar results for local relapse free survival. Benefit from the addition of CON to RT was seen in patients with tumour necrosis (log rank $P = 0.001$) but not in those without (log rank $P = 0.24$). For comparison and because of their wide use as hypoxia markers, the ability of CA-IX and Glut-1 to predict benefit from hypoxia modification was also studied. In 63 patients with CA-IX score = 0, there was no significant difference in five-year OS (41% for RT alone versus 51% RT + CON, log rank

Table 3
Multivariate Cox regression stratified by necrosis. All covariates included in the model are also tabulated (note the stratum specific treatment variables, strata:treatment interaction $P = 0.007$ unadjusted, $P = 0.001$ adjusted).

Variable	N_{MV}	HR_{MV}	95% CI	*P*
Gender				0.32
Male (reference)	177			
Female	43	1.28	0.79–2.05	
Age (years)*	220	1.05	1.03–1.08	<0.0001
Stage				0.16
T1 (reference)	20			
T2	148	2.16	0.94–5.00	
T3	42	1.55	0.60–4.00	
T4a	10	2.13	0.64–7.09	
TURBT				0.63
Complete (reference)	90			
Partial	70	0.99	0.64–1.53	
Biopsy	60	1.22	0.78–1.93	
pTis				0.05
Absent (reference)	162			
Present	58	1.52	1.01–2.29	
Hb (g/L)*	220	0.89	0.77–1.00	0.06
Necrosis absent:treatment				0.08
RT (reference)	55			
RT + CON	50	1.64	0.95–2.85	
Necrosis present:treatment				0.002
RT (reference)	57			
RT + CON	58	0.43	0.25–0.73	

Abbreviations: HR, hazard ratio; MV, multivariate; TURBT, transurethral resection of the bladder tumour; pTis, carcinoma *in situ*; Hb, haemoglobin.
BCON patients with all clinicopathologic variables recorded $n = 220$; 2 cases Hb and 9 cases TURBT not reported.
* Variables marked with a * were entered as continuous variables and hence the HR is for a unit increase in the variable.

$P = 0.52$) (Fig. 4C). In 126 with CA-IX score >0, the five-year OS was 35% for RT alone versus 51% for RT + CON (log rank $P = 0.03$) (Fig. 4D). Stratification of 185 patients based upon Glut-1 score revealed no significant differences in survival between trial arms (Fig. 4E and F). Given its potential to affect tumour hypoxia, patients were also stratified according to haemoglobin levels (using previously determined cutoffs [27]); no significant differences in survival between trial arms were observed (Supplementary Fig. S2).

Multivariate analyses showed that only necrosis was a significant independent predictor of benefit from CON. The risk of death was lower when a patient received RT + CON compared to RT alone when necrosis was present (HR 0.43, 95% CI 0.25–0.73, $P = 0.002$). This trend was not observed when necrosis was absent (HR 1.64, 95% CI 0.95–2.85, $P = 0.08$) (Table 3). Tests for heterogeneity in treatment effect by necrosis strata were significant ($P = 0.007$ unadjusted, $P = 0.001$ adjusted). A test for interaction also showed there was a significant treatment effect by necrosis strata for local relapse free survival ($P = 0.003$ unadjusted, $P = 0.0003$ adjusted). Supplementary Tables S3 and S4 show the comparative data for CA-IX ($P = 0.08$ unadjusted, $P = 0.20$ adjusted) and Glut-1 ($P = 0.75$ unadjusted, $P = 0.61$ adjusted) respectively. Haemoglobin also showed no significant evidence for treatment interaction ($P = 0.29$ unadjusted).

Tumour necrosis was significantly associated with TURBT (chi-square $P = 0.03$), growth pattern (chi-square $P = 0.004$), and low Hb concentration (Mann Whitney *U* $P = 0.0006$). There was a greater likelihood of detecting tumour necrosis with increasing extent of surgical resection. Intra-tumour heterogeneity in necrosis was low in mixed TURBT samples; the mean probability that two randomly chosen blocks from a tumour both showed necrosis or otherwise was 0.83 (weighted mean 0.80; 46 cases, 2–8 blocks) (Supplementary Table S5). Solid tumours were more likely to be necrotic than papillary or mixed. Tumour necrosis was not associated with tumour stage (chi-square $P = 0.14$) but was associated with CA-IX ($n = 189$; Mann Whitney *U* $P < 0.0001$) and Glut-1 ($n = 185$; Mann Whitney *U* $P = 0.002$) expression (Supplementary Table S4). Ki-67 showed no significant association with necrosis ($n = 92$, Mann Whitney *U*, $P = 0.68$; Supplementary Fig. S3).

Discussion

Necrosis was the only histopathological tumour feature with independent prognostic ability. This is the first confirmation of the findings of a study reporting necrosis to be an independent adverse prognostic factor in high grade and invasive bladder cancer [13]. In support, Zigeuner et al. [28] showed necrosis predicts clinical outcomes in 1425 patients (from 13 worldwide centres) undergoing radical surgery for urothelial cell carcinoma of the upper urinary tract [28]. Contrary to previous reports, putative hypoxia markers CA-IX and Glut-1 [11] did not significantly indicate a poor prognosis possibly due to differences in quantification technique and biological variation between samples. The use of TMAs might have led to sampling error and could be a limitation of our study.

Other independent adverse prognostic factors were older patient age and concurrent pTis. Difficulties in staging in the absence of cystectomy may explain why tumour stage was not a prognostic risk factor. Established prognostic risk factors for bladder cancer are tumour grade, stage and the presence of concurrent CIS [26]. As a result of heterogeneity in bladder cancer behaviour, these factors are insufficient to identify patients with a high risk of death following radical cystectomy or RT. We propose that necrosis is used for individualised patient prognostication and clinical decision making. It is simple to measure and could easily be incorporated into routine histopathological examination. In our multivariate analysis, classification as absent or present (HR 2.1, $P = 0.04$) compared favourably with classification as presence and amount (HR 1.9, $P = 0.04$) as reported by Ord et al. [13].

Necrosis was significantly associated with increasing extent of TURBT, a solid growth pattern and low Hb levels. Association of necrosis with low Hb supports its link to chronic hypoxia, as low Hb levels reduce blood oxygen-carrying capacity. Low Hb has also been associated with a poor response to radiation [27]. The lack of association between necrosis and tumour stage (as previously reported [13]) may be due to difficulties in staging from TURBT. Association of necrosis with extent of TURBT might relate to an improved chance of detection in larger tissue samples. Patients not undergoing complete tumour debulking or cystectomy might have a small risk of misclassification due to smaller sample size. TURBT might be related to tumour volume and so a confounding factor. Unfortunately tumour volume data were not collected in the BCON trial so we cannot rule out this possibility.

Necrotic tumours had higher expression of CA-IX and Glut-1, consistent with it demarcating areas of chronic hypoxia. Expression of CA-IX or Glut-1 is associated with a poor prognosis following RT in head and neck cancer [29,30]. We theorised that necrotic tumours would have a high proliferation with rapid cell turnover leading to outstripping of their oxygen supply. The lack of increased Ki-67 expression in necrotic tumours suggests hypoxia may develop as a result of poor distribution or perfusion of vasculature rather than high cell proliferation. Despite the strong association of CA-IX with tumour necrosis many CA-IX positive tumours (40/84, 47.6%) had no evident necrosis. In these tumours, CA-IX may be under the control of hypoxia-independent factors. Sampling bias may explain the lack of CA-IX labelling in the presence of necrosis (19/105, 18.1%). This trend was less clear for Glut-1, as this marker was less specific.

Clinical trials using hypoxia-modifying therapies have been ongoing since the late 1960s. Meta-analysis of all randomised

clinical trials of hypoxia-modification involving RT showed significant improvement in local control [31]. However, it is difficult to evaluate the full benefit of treatment in unstratified study populations. We have shown for the first time that tumour necrosis independently predicts benefit from hypoxia-modifying therapy. Although CA-IX and Glut-1 have been associated with a poor prognosis in bladder cancer patients [11], only CA-IX predicted treatment benefit; which was subsequently lost upon multivariate analysis. It is probable that despite being strongly linked to necrosis, they lack hypoxia-specificity, technical and quantitative precision. Therefore, a prospective trial should be considered using necrosis to select patients for RT plus hypoxia-modifying therapy versus a novel intervention in those with no tumour necrosis. BCON is currently in phase IV evaluation in UK; it is routine treatment for invasive and high grade bladder cancer in Mount Vernon Hospital, Middlesex, UK and at least two other UK hospitals. This is an efficacious treatment and the use of necrosis as a companion biomarker strengthens the case for its widespread uptake. Use of necrosis to select patients for BCON can improve five-year OS by 22%.

Conflict of Interest Statement

We declare no conflicts of interest.

Role of the Funding Source

Study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.05.017.

References

[1] Grossman HB, Natale RB, Tangen CM, et al. Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. N Engl J Med 2003;349:859–66.
[2] Dunst J, Rodel C, Zietman A, Schrott KM, Sauer R, Shipley WU. Bladder preservation in muscle-invasive bladder cancer by conservative surgery and radiochemotherapy. Semin Surg Oncol 2001;20:24–32.
[3] Rodel C, Grabenbauer GG, Kuhn R, et al. Combined-modality treatment and selective organ preservation in invasive bladder cancer: long-term results. J Clin Oncol 2002;20:3061–71.
[4] Hoskin PJ, Rojas AM, Bentzen SM, Saunders MI. Radiotherapy with concurrent carbogen and nicotinamide in bladder carcinoma. J Clin Oncol 2010;28:4912–8.
[5] Overgaard J, Eriksen JG, Nordsmark M, Alsner J, Horsman MR. Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. Lancet Oncol 2005;6:757–64.
[6] Kaanders JH, Wijffels KI, Marres HA, et al. Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. Cancer Res 2002;62:7066–74.
[7] Rischin D, Hicks RJ, Fisher R, et al. Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: a substudy of Trans-Tasman Radiation Oncology Group Study 98.02. J Clin Oncol 2006;24:2098–104.
[8] Toustrup K, Sorensen BS, Lassen P, Wiuf C, Alsner J, Overgaard J. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. Radiother Oncol 2012;102:122–9.
[9] Toustrup K, Sorensen BS, Nordsmark M, et al. Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. Cancer Res 2011;71:5923–31.
[10] Janssens GO, Rademakers SE, Terhaard CH, et al. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. J Clin Oncol 2012;30:1777–83.
[11] Hoskin PJ, Sibtain A, Daley FM, Wilson GD. GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON. Br J Cancer 2003;89:1290–7.
[12] Palit V, Phillips RM, Puri R, Shah T, Bibby MC. Expression of HIF-1alpha and Glut-1 in human bladder cancer. Oncol Rep 2005;14:909–13.
[13] Ord JJ, Agrawal S, Thamboo TP, et al. An investigation into the prognostic significance of necrosis and hypoxia in high grade and invasive bladder cancer. J Urol 2007;178:677–82.
[14] Bussink J, van der Kogel AJ, Kaanders JH. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. Lancet Oncol 2008;9:288–96.
[15] Iakovlev VV, Pintilie M, Morrison A, Fyles AW, Hill RP, Hedley DW. Effect of distributional heterogeneity on the analysis of tumor hypoxia based on carbonic anhydrase IX. Lab Invest 2007;87:1206–17.
[16] Thrall DE, Rosner GL, Azuma C, McEntee MC, Raleigh JA. Hypoxia marker labelling in tumor biopsies: quantification of labelling variation and criteria for biopsy sectioning. Radiother Oncol 1997;44:171–6.
[17] Remzi M, Haitel A, Margulis V, et al. Tumour architecture is an independent predictor of outcomes after nephroureterectomy: a multi-institutional analysis of 1363 patients. BJU Int 2009;103:307–11.
[18] Kruger S, Noack F, Bohle A, Feller AC. Histologic tumor growth pattern is significantly associated with disease-related survival in muscle-invasive transitional cell carcinoma of the urinary bladder. Oncol Rep 2004;12:609–13.
[19] Harris AL. Hypoxia – a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38–47.
[20] Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, et al. Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 2012;11.
[21] Mesker WE, Liefers GJ, Junggeburt JM, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I–II colon cancer patients. Cell Oncol 2009;31:169–78.
[22] Courrech Staal EF, Wouters MW, van Sandick JW, et al. The stromal part of adenocarcinomas of the oesophagus: does it conceal targets for therapy? Eur J Cancer 2010;46:720–8.
[23] de Kruijf EM, van Nes JG, van de Velde CJ, et al. Tumor–stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat 2011;125:687–96.
[24] Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour–stroma ratio in triple-negative breast cancer. Eur J Surg Oncol 2012;38:307–13.
[25] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
[26] Youssef RF, Lotan Y. Predictors of outcome of non-muscle-invasive and muscle-invasive bladder cancer. Sci World J 2011;11:369–81.
[27] Hoff CM, Hansen HS, Overgaard M, et al. The importance of haemoglobin level and effect of transfusion in HNSCC patients treated with radiotherapy – results from the randomized DAHANCA 5 study. Radiother Oncol 2011;98:28–33.
[28] Zigeuner R, Shariat SF, Margulis V, et al. Tumour necrosis is an indicator of aggressive biology in patients with urothelial carcinoma of the upper urinary tract. Eur Urol 2010;57:575–81.
[29] Koukourakis MI, Bentzen SM, Giatromanolaki A, et al. Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. J Clin Oncol 2006;24:727–35.
[30] De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S. The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/− chemotherapy. BMC Cancer 2005;5:42.
[31] Horsman MR, Van der Kogel AJ. Therapeutic approaches to tumour hypoxia. In: Joiner M, Van der Kogel AJ, editors. Basic clinical radiobiology. London: Hodder Arnold; 2009. p. 233–45.